# Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery

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## Abstract

Very little is known about the culturability and viability of mycobacteria following their phagocytosis by macrophages. We therefore studied populations of the avirulent 'Academia' strain of *Mycobacterium tuberculosis* isolated from murine peritoneal macrophage lysates several days post-infection in vivo. The resulting bacterial suspensions contained a range of morphological types including rods, ovoid forms and coccoid forms. Bacterial viability measured using the MPN method (dilution to extinction in liquid medium) was often much higher than that measured by CFU (plating on solid medium). Viability in the MPN assay was further enhanced when the *Micrococcus luteus* protein, Rpf, was incorporated into the liquid culture medium at picomolar concentrations. Rpf is an example of a family of autocrine growth factors found throughout the high G+C cohort of Gram-positive bacteria including *M. tuberculosis*. *M. tuberculosis* cells obtained from macrophages had altered surface properties, as compared with bacteria grown in vitro. This was indicated by loss of the ability to adsorb bacteriophage DS6A, a reduced tendency to form clumps, acquisition of ethidium bromide stainability following heat treatment, and loss of Rpf-mediated resuscitation following freezing and thawing. These results indicate that a proportion of 'unculturable' *M. tuberculosis* cells obtained from macrophages is either injured or dormant and that these cells may be recovered or resuscitated using Rpf in liquid medium. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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## 1. Introduction

In common with several other bacterial pathogens, mycobacteria have evolved mechanisms to ensure their survival inside macrophages [1,2]. In the case of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), this results in a chronic form of the disease. Bacteria may persist in vivo for long periods [1,3], by passing into a latent or dormant state [4–6]. Dormancy has been defined as a *reversible* state of low metabolic activity [7–9]. Dormant cells may not be 'alive' in the sense of being able to form a colony when plated on a suitable growth medium. However, they are not 'dead', since they can regain colony-forming ability following a period of resuscitation,

which may require carefully defined nutritional conditions [9].

Although the existence of latent *M. tuberculosis* infections has long been recognised, the precise nature of the latent state is not well understood [6]. The extent to which suppression of bacterial growth by the host immune system and intrinsic dormancy of the bacteria themselves contribute to latency is still a matter of conjecture. Attempts to gain meaningful insights into the latent state, by examining the ability of mycobacteria to produce dormant forms, either in culture or inside macrophages, have met with only limited success. Several model systems have been developed in attempts to mimic the in vivo state (see [6] for a review). For example, Khomenko obtained filterable forms of a bacillus from TB patients, but it is unclear whether these represent dormant bacteria, or injured, moribund, or even dead cells [10,11]. Wayne used the term 'dormant' to describe a population of *M. tuberculosis* cells observed after the transition from an aerobic to an anaer-

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obic state [12,13]. However, the significance of this state provoked in vitro for the persistence of mycobacteria in vivo remains to be clarified. In the Cornell dormancy model [6] infected mice are treated with antibiotics, after which the number of cultivable cells of *M. tuberculosis* extractable from organs falls to a value indistinguishable from zero. However, quantitative PCR revealed the presence of DNA equivalent to about $10^5$ organisms per gram tissue [14]. After several months of latency, disease symptoms and cultivable organisms reappear, suggesting the existence of some form of dormancy.

The interactions that occur between *M. tuberculosis* and monocytes and macrophages have been studied extensively over the years. Many reports have dealt with different aspects of the persistence and growth of both virulent and avirulent strains in macrophages of either murine or human origin [15–21]. However, surprisingly little has been published about the effect of persistence within the macrophage environment on bacterial viability. In one of these reports [19] measurements were made of both total count and viable count (CFU) of *M. tuberculosis* within human monocyte-derived macrophages 6 days post-infection. Inspection of these data leads to the conclusion that for some macrophage cultures, a significant proportion (70–90%) of the bacteria obtained was unable to form colonies. They might be injured, moribund, or even dead following persistence within the inimical macrophage environment, where they are potentially exposed to reactive nitrogen intermediates, as well as a multitude of acid hydrolases following phagosome–lysosome fusion [22,23]. Alternatively, they might conceivably be in a latent or dormant state.

*Micrococcus luteus* is a Gram-positive non-sporulating bacterium, related to mycobacteria, in which the existence of a dormant state has been clearly established [8,24–27]. Viable cells of *M. luteus* secrete a proteinaceous growth factor (Rpf) which promotes the resuscitation of dormant, non-growing cells in liquid medium to yield normal, viable, colony-forming bacteria. Resuscitation experiments were performed using the MPN (most probable number) assay, which allows one to estimate the number of viable cells, by cultivation in liquid medium supplemented with Rpf [28]. Rpf is also required for the growth of extensively washed viable cells of *M. luteus* in a minimal liquid medium, indicating that it should be regarded as a bacterial growth factor, or cytokine [28]. Rpf also stimulates the growth of viable cells of both virulent and avirulent strains of *M. tuberculosis* [28]. This organism contains five genes encoding Rpf-like proteins [29], which raises the possibility that one or more of these gene products may also be involved in controlling growth and latency of *M. tuberculosis* in vivo.

The aims of the present study were to characterise *M. tuberculosis* cells obtained from macrophages with respect to their surface properties and their culturability, and to establish whether they respond to Rpf.

## 2. Materials and methods

### 2.1. Bacterial strains

Two strains of *M. tuberculosis* were employed in this investigation, the avirulent 'Academia' strain [30] and the virulent H37Rv strain. The Academia strain was obtained from the Phthysiopulmonology Center (Moscow). Bacteria were maintained on Lowenstein–Jensen agar slopes at 37°C and grown for 15 days at 37°C in liquid Sauton's medium supplemented with 0.6% (w/v) glycerol, and with albumin, glucose and NaCl (ADC) [31]. Aggregate-free cell suspensions were obtained by brief sonication (20 s) followed by centrifugation of the resulting suspension, firstly at $2000 \times g$ for 5 min (pellet discarded) and secondly at $10\,000 \times g$ for 20 min. The pellet from the second centrifugation was resuspended in RPMI 1640 medium (Gibco) and bacteria were counted in a Neubauer chamber. Mycobacterial viability (culturability [9]) was assessed by determining the number of CFU and the MPN as described below.

### 2.2. In vivo infection

BALB/c mice were infected with 0.2 ml of a suspension of *M. tuberculosis* in saline (0.9% NaCl) supplemented with 0.01% Tween-80 (ca. $10^8$ cells per animal) by intraperitoneal injection. The inoculum was grown in Sauton's medium in vitro. After 4–14 days, ascitic fluid was taken from the mice and the macrophages were washed with RPMI 1640 (Gibco) by centrifugation at $500 \times g$ before seeding them in 1 ml RPMI in 2-ml wells (Costar) (initial cell density ca. $10^5$ cells per well). The macrophages were then grown in RPMI containing 5% foetal calf serum (Gibco) containing 100 U gentamicin (Sigma) per ml at 37°C, in an atmosphere containing 5% $CO_2$. During this 1–2-day conditioning phase, they reached confluence, corresponding to approximately $5 \times 10^5$ macrophages per well. Before harvesting, the macrophage monolayer was washed twice with RPMI to remove macrophage debris and any extracellular bacteria. The infected macrophages were suspended in 1 ml Sauton's medium and intracellular bacteria were obtained by passing the cell suspension through a 23-gauge syringe needle. Macrophage lysis was checked microscopically. Samples of the resulting mycobacterial suspensions were serially diluted in Sauton's medium, and used for determination of viability by measuring CFU and MPN, as described below.

For the second passage, mycobacterial suspensions obtained from infected macrophages after their conditioning as described above were employed to infect mice by intraperitoneal injection at a density of ca. $10^4$ bacterial cells per mouse. The inoculum size was limited by the number of bacterial cells obtained from the first passage. Macrophages were subsequently isolated from the ascitic fluid and conditioning, macrophage lysis and determination of

bacterial viability were carried out as above. The macrophage seeding density during the conditioning phase was similar to that employed for the first passage.

To isolate small coccoid forms, the mycobacterial suspension obtained from macrophages was passed through a 0.45 μm filter (Millipore) and the filtrate was examined microscopically. Viability was tested by plating and by MPN determination with and without Rpf (see below).

## 2.3. Bacteriophage adsorption

Bacteriophage DS6A [32–35] lysates were obtained by infecting a suspension of $10^6$ CFU of a phage-sensitive, clinical isolate of *M. tuberculosis* at a multiplicity of infection (MOI) of 50. After allowing the bacteria to grow for 24 h at 37°C, they were disrupted by adding chloroform (1%) to the suspension and shaking for 30 min. Liberated phages were sedimented by ultracentrifugation ($200\,000 \times g$) and purified by sucrose density gradient centrifugation according to Timme and Brennan [36]. Phage particles were harvested from the gradient and the titre was determined by counting under the electron microscope, using latex particles, (87 nm diameter; Dow Chemical Co.) as an internal standard.

For electron microscopy, *M. tuberculosis* cells grown on agar-solidified, ADC-supplemented Sauton's medium were washed twice with, and resuspended in saline (0.9% NaCl). Bacteriophage DS6A particles were added at a MOI of $10^3$. After incubation for 2 h at 37°C with gentle shaking, the mycobacteria were harvested by centrifugation, resuspended in 0.1 M Na phosphate buffer, pH 7, and fixed by the addition of 2% glutaraldehyde. Fixed cells were stained with 1% uranyl acetate by using colloidon support films for negative contrast and examined using a Hitachi-300 electron microscope. *M. tuberculosis* cells that had not been exposed to bacteriophage were used as a control. A minimum of 5 fields with about 100 bacteria in each was counted.

## 2.4. Isolation of M. luteus Rpf

The Rpf protein of *M. luteus* (histidine-tagged recombinant form) was obtained as described by Mukamolova et al. [28]. The purified protein was stored in 10 mM Tris–HCl pH 7.4 containing 50% glycerol at −20°C and the protein concentration was determined spectrophotometrically as described previously [28].

## 2.5. Assessment of cell viability (culturability)

*M. tuberculosis* cells were obtained from infected macrophages as described above. Bacterial suspensions were serially diluted in Sauton's medium, using a fresh pipette for each dilution and plated in triplicate on agar-solidified, ADC-supplemented Sauton's medium. The number of CFU was counted after 3 and 6 weeks. Duplicate 100-μl

samples were plated and the detection limit was therefore $5 \times 10^0$ CFU ml$^{-1}$.

The same serially diluted samples were employed for MPN assays. Using a fresh pipette tip each time, 10 samples (100 μl) were taken from each dilution and added to 10 replicate 10-ml Pyrex screw-capped tubes, each containing 2 ml Sauton's medium supplemented with ADC. Five of these tubes contained Rpf, previously added to a final concentration of between 27 and 68 pmol l$^{-1}$. The tubes were incubated at 37°C without shaking for 2 months. Tubes with visible growth of *M. tuberculosis* were counted as positive. MPN values were calculated using standard statistical methods [37].

In five experiments, the total number of *M. tuberculosis* cells was determined microscopically using a haemocytometer. A minimum of 100 bacteria was counted and the S.D. on the total counts was not in excess of 20%.

For the control experiments reported in Table 1, the Academia strain was grown in vitro in the presence of 0.05% Tween-80. Cell suspensions were passed though a 23-gauge syringe needle to disperse aggregates before determination of total count, CFU and MPN ± Rpf (68 pmol l$^{-1}$).

In some experiments, cells obtained from macrophages were subjected to one cycle of freezing (−20°C for 24–48 h) and thawing to ambient temperature. Their viability was determined by measuring the numbers of CFU and the MPN (±Rpf) as described above. To assess the integrity of the cell membrane following heat treatment (65°C for 30 min), bacteria were stained by incubation at room temperature for 5 min in their growth medium containing ethidium bromide (1 μM). They were then examined by fluorescence microscopy as described below. Cells of *M. tuberculosis* growing in vitro are not stained after heating, whereas cells obtained from macrophages are.

## 2.6. Assessment of respiratory activity

This was determined by using the fluorescent redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride-Polysciences, Eppelheim, Germany) as described by Kaprelyants and Kell [38]. Briefly, the bacteria were incubated in the presence of 4 mM CTC (freshly prepared in 10 mM Na phosphate buffer pH 7.0) for 30 min at 37°C and fluores-

Table 1
Comparison of *M. tuberculosis* (Academia) total counts and viable counts measured by CFU and MPN methods

| | Experiment | | |
| --- | --- | --- | --- |
| | 1 | 2 | 3 |
| Age of culture (days) | 20 | 45 | 61 |
| Total count | $1.48 \times 10^8$ | $2.4 \times 10^9$ | $5 \times 10^7$ |
| CFU | $1.42 \times 10^8$ | $1.05 \times 10^9$ | $1.24 \times 10^6$ |
| MPN | $1.4 \times 10^8$ | $1.7 \times 10^9$ | $2.8 \times 10^7$ |
| MPN+Rpf | $1.4 \times 10^8$ | $2.2 \times 10^9$ | $4.6 \times 10^7$ |

Values are per ml culture.

cence was monitored with a Leica fluorescence microscope with excitation at 530–550 nm.

## 3. Results

### 3.1. Recovery of viable cells from macrophages

Macrophages taken from infected mice were examined microscopically 4–14 days post-infection to determine the proportion of infected cells. Macrophage populations contained between 20 and 40% of infected cells. Microscopic examination revealed that each phagocyte contained between two and four acid-fast bacteria. This level of infection is similar to that observed by others working with avirulent strains of M. tuberculosis [19,22,39]. The number of passages did not significantly affect the percentage of phagocytic macrophages, nor the number of acid-fast bacteria within infected cells.

The total mycobacterial count (determined microscopically) of suspensions obtained from macrophages on five separate occasions was quite uniform (i.e. ca. $10^6$ organisms ml$^{-1}$). However, the number of viable CFU in these suspensions was extremely variable from one experiment to another (from zero to $10^4$ CFU ml$^{-1}$). The total count and the viable count thus differed by between two and six orders of magnitude in different experiments. The great majority of mycobacteria obtained from macrophages were unable to form colonies under the conditions employed here.

### 3.2. Comparison of viable count determined by MPN and CFU

The viable counts of cell suspensions derived from macrophages were compared using the CFU and MPN assays. This methodology was employed previously to detect dor-
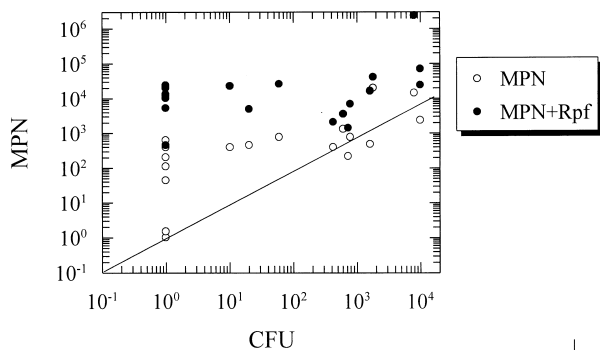


Fig. 1. Relationship between the number of viable M. tuberculosis cells obtained from macrophages as judged by CFU (plating on agar-solidified Sauton's medium) and by MPN (assay in liquid medium). Each point represents a separate experiment. Open symbols, Rpf absent; closed symbols, recombinant histidine-tagged Rpf present at a concentration of 27–68 pmol l$^{-1}$. All points above the diagonal on the figure show a higher viable count in liquid medium (MPN) than on solid medium (CFU).

mant cells of M. luteus and resuscitate them from this 'unculturable' state [25]. The MPN method allows numerical estimation of number of viable bacteria by their cultivation in liquid medium at high dilutions. Provided that the MPN medium supports resuscitation as well as growth, a 'non-culturable' cell will score as viable, in contrast to its behaviour as judged by direct plating [9]. The results of 18 independent experiments are summarised in Fig. 1. These data encompass viable counts made after one or two passages (see above). Since dormancy (non-culturability) in M. luteus is only observed after prolonged incubation in stationary phase in vitro [24,27], we reasoned that there might be an increase in the proportion of non-culturable cells of M. tuberculosis after a second passage. However, there was no evidence of this. The number of passages was not correlated with the viable count determined by either CFU or MPN. For samples with a high number of CFU (ca. $10^4$ CFU ml$^{-1}$) there was an approximate correspondence between the viable counts as determined by the MPN and CFU assays. However, for samples containing low numbers of CFU, the viable count as determined by the MPN assay was generally substantially higher than that obtained by the CFU assay. Indeed, some samples in which essentially no viable cells were detectable by plating, gave viable counts by MPN of between $10^2$ and $10^3$ bacteria ml$^{-1}$ (Fig. 1, open circles).

To rule out the possibility that the observed differences between MPN and CFU were due to a systematic error (e.g. MPN estimates always 10–100-fold higher than CFU counts, or MPN estimates always erroneous at low CFU counts) several control experiments were performed. The total bacterial count of aggregate-free suspensions of bacteria grown in vitro (see Section 2) was determined microscopically. Samples of these suspensions were serially diluted for CFU and MPN determination, as described in Section 2, using Sauton's medium, solidified with agar as appropriate, throughout. The results of three representative experiments (Table 1) show that for bacteria actively growing in vitro, viability was the same when measured by plating or by the MPN method. Moreover, the viable count was comparable to the total count. However, as cultures were left to age a decreasing proportion of the bacteria were able to form colonies. Viability was enhanced when measured by the MPN method and was almost the same as the total count when the MPN was determined in the presence of Rpf.

### 3.3. Effect of Rpf on viable count determined by the MPN assay

Viable counts of cell suspensions derived from macrophages were also measured by the MPN method in the presence of Rpf. These data are also summarised in Fig. 1 (closed circles), in relation to the number of CFU in the various macrophage lysates. In the presence of Rpf, most of the samples yielded MPN counts of between $10^3$ and
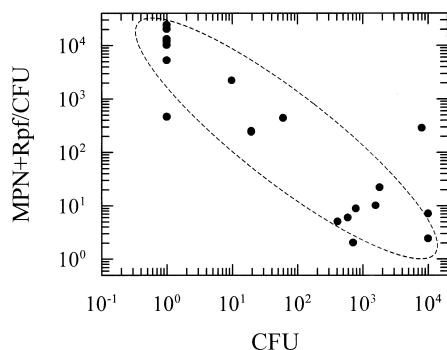
Fig. 2. Rpf enhances the viable count of *M. tuberculosis* suspensions obtained from infected macrophages. MPN was determined in the presence of Rpf (27–68 pmol $l^{-1}$) and the MPN/CFU ratio is related to the viable count on solid medium (CFU). The ellipse drawn on the figure highlights the inverse relationship between MPN/CFU and CFU.

$8 \times 10^3$ organisms $ml^{-1}$, irrespective of the number of CFU present. Rpf stimulated the MPN count in all samples, with the majority of data points in the 10–100-fold stimulation range. The ratio of the MPN counts determined with Rpf to the number of CFU expressed as a function of the number of CFU (Fig. 2) revealed an inverse correlation between two methods of estimation of bacterial viability. Rpf had a maximal effect on cell viability with samples that yielded the lowest CFU values.

Growth stimulation by Rpf can also be measured by monitoring the apparent lag phase of bacterial cultures [28]. The apparent lag phase for growth of *M. tuberculosis* obtained from macrophages was substantially reduced in the presence of Rpf. During the course of the 18 experiments summarised in Figs. 1 and 2, we monitored the time to appearance of visible bacterial growth (small aggregates that formed on the bottom of the tube) in all of the MPN tubes inoculated with $10^4$ bacteria (total count). This was detected after 18 ($\pm$2) days in the presence of Rpf and after 32 ($\pm$5) days in its absence.

Virulent strains of *M. tuberculosis* persist and grow more actively in murine macrophages than do avirulent strains [16,18,19]. We therefore undertook two experiments (one passage) using the virulent H37Rv strain of *M. tuberculosis* to determine whether it showed similar behaviour to the avirulent Academia strain (Table 2). Bacterial suspensions derived from macrophages 6 days post-infection had relatively high numbers of CFU and the MPN values were similar. As was observed with the avir-
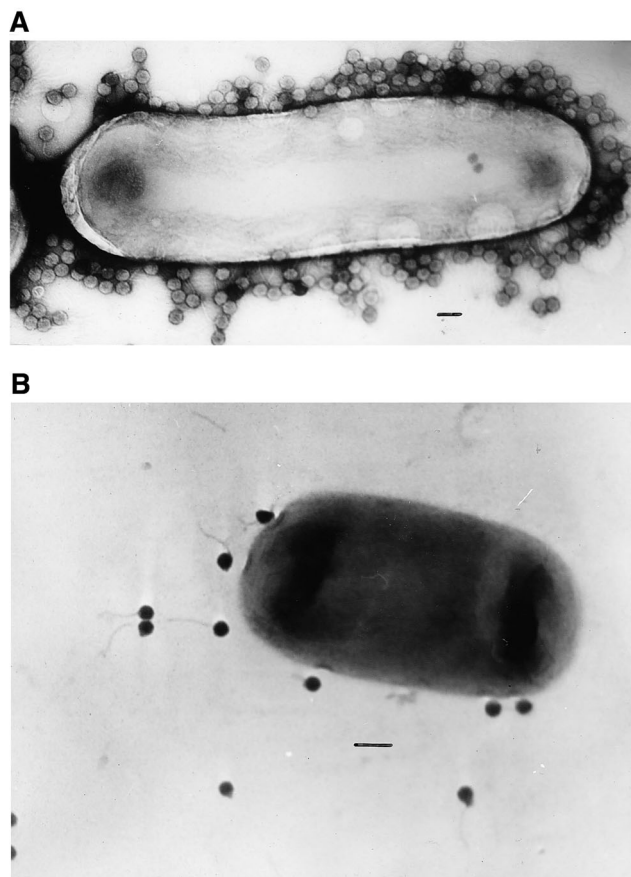


Fig. 3. Binding of bacteriophage DS6A to *M. tuberculosis* cells. A: Bacteria growing exponentially in vitro: more than 100 adsorbed phages per cell. B: Bacteria obtained from macrophages (first passage, in vivo): 2–5 adsorbed phages per cell. The bar marker represents 100 nm.

ulent Academia strain, Rpf stimulated the viable count as determined by the MPN method. For the H37Rv strain, an increase of between 10- and 20-fold was observed.

### 3.4. M. tuberculosis cells obtained from macrophages do not actively respire

The respiratory activity of *M. tuberculosis* cells (Academia strain) obtained from macrophages was investigated using the fluorescent redox dye, CTC [38]. Fluorescence microscopy revealed that none of the bacteria observed (four independent experiments; >100 cells in each) was undergoing active respiration. In contrast, almost 100% of cells from a population growing actively in vitro showed positive staining with CTC. These results indicate that *M. tuberculosis* cells obtained from macrophages are not metabolically active, in accordance with data obtained using the Wayne dormancy model [40].

### 3.5. Morphology and surface properties of cells obtained from macrophages

*M. tuberculosis* cells (Academia strain) obtained from

Table 2
Rpf stimulates *M. tuberculosis* H37Rv viability measured by the MPN method

|  | Experiment 1 | Experiment 2 |
| --- | --- | --- |
| CFU | $5 \times 10^3$ | $1.2 \times 10^3$ |
| MPN | $3.3 \times 10^3$ | $5.4 \times 10^3$ |
| MPN+Rpf | $3.5 \times 10^4$ | $9 \times 10^4$ |
| Ratio: MPN+Rpf/MPN | 10.6 | 16.7 |

Values are per ml macrophage lysate.

macrophages were compared with cells grown in vitro by light microscopy. The bacteria obtained from macrophages fell into three distinguishable morphological classes (short rods, ovoid forms and coccoid forms). The proportions of the three different types (determined using the light microscope) and their approximate dimensions (determined by electron microscopy, using the same batches of mycobacteria) changed during exposure to the intracellular macrophage environment. During the first passage in vivo the normal rod-shaped cells were converted into ovoid forms (diameter $1.2 \pm 0.4$ µm) (70–80%) and short rods (length $1.2 \pm 0.2$ µm; diameter $0.6 \pm 0.2$ µm) (20–30%) (Fig. 3). After the second passage, about 90% of the cells present in populations obtained from macrophages were even smaller coccoid forms. All these growth forms were stainable using Ziehl–Neelsen reagent. However, in contrast to the rods and ovoid forms, the small coccoid forms, obtained by filtration through a 0.45 µm filter, were not cultivable. They failed to form colonies on agar-solidified Sauton's medium and did not grow in liquid Sauton's medium, even in the presence of Rpf (data not shown).

Evidence suggesting that both the ovoid and coccoid forms obtained from macrophages have an altered cell surface was obtained by examining their ability to adsorb the *M. tuberculosis*-specific bacteriophage, DS6A [32,34,35]. Cells growing exponentially in vitro adsorbed more than 100 phages per cell whereas cells obtained from macrophages adsorbed significantly fewer phage particles (53% adsorbed between 10 and 100 phages, 45% adsorbed between 2 and 5 phages and 2% adsorbed no phages at all; Fig. 3). Several additional observations lend support to the conclusion that there are significant alterations to the surface of the mycobacterial cell envelope following exposure to the intracellular macrophage environment. These bacteria were less adherent and therefore less susceptible to clumping than cells grown in vitro, as revealed by microscopic examination. They were stained by ethidium bromide after heating at 65°C for 30 min, whereas cells grown in vitro could not be stained after heat treatment (data not shown). Moreover, Rpf-mediated recovery/resuscitation of cells obtained from macrophages was negligible after freezing and thawing, whereas this treatment does not affect the culturability of cells grown in vitro.

## 4. Discussion

When *M. tuberculosis* cells are ingested by macrophages, most of the bacteria are found in fused phagolysosomes within hours of infection [22]. Subsequently, many organisms escape. The majority of cells appear to have been extruded into tightly apposed membrane vesicles, whereas some cells may appear free in the cytoplasm [22]. Virulent strains of *M. tuberculosis* are able to resist killing by reactive nitrogen intermediates [15,17] and they multiply within the phagocyte [16,18]. They may, however suffer

transient injury. Avirulent strains are generally less well able to proliferate in phagocytes than virulent strains, although they can persist (or even multiply slowly) in human monocytes [21], human macrophages [39] and murine macrophages [19,22].

There have been very few studies to date concerning the physiological properties of mycobacterial cells obtained from macrophages. Evidence suggesting that *M. tuberculosis* cells lose culturability after phagocytosis by human macrophages has been published [19] although the authors do not draw this specific conclusion from their data. In the present work, we found that the total count of acid-fast bacteria in macrophage lysates exceeded the number of CFU by several orders of magnitude. Clearly, a substantial proportion of the bacteria obtained from macrophages was not cultivable.

There may be several reasons for this. For example, non-specific injury might occur within the inimical macrophage environment, rendering the cells delicate, or moribund, or even dead. Alternatively, their behaviour might reflect a programmed response (akin to dormancy) set in train as a result of exposure to the macrophage environment, which might be related to the well-known phenomenon of latency. Indeed, the non-culturability of acid-fast *M. tuberculosis* obtained from lesions resected from human lung tissue has been known for decades (e.g. [41]), and attempts to 'resuscitate' such non-cultivable cells were unsuccessful [42].

The *M. tuberculosis* cells obtained from murine macrophages were severely impaired in their capacity to adsorb bacteriophage DS6A, indicating loss (or modification) of the bacteriophage receptor at the cell surface. Other changes following phagocytosis by macrophages, such as the progressive adoption of an ovoid and then a coccoid morphology, and a reduced tendency to clump, are also indicative of the occurrence of alterations to the mycobacterial cell envelope. Moreover, increased fragility would seem to be indicated by the loss of Rpf-mediated resuscitation following freezing and thawing and the acquisition of ethidium bromide stainability following heat treatment. All of these properties are consistent with the occurrence of non-specific injury resulting from exposure to the intracellular macrophage environment. Such organisms might therefore be particularly sensitive to the detergents (such as SDS and Tween-80) that are tolerated by bacteria growing in vitro and that are commonly employed for their isolation.

Nevertheless, the possible existence of a state akin to dormancy following exposure to the intracellular macrophage environment, even for a comparatively short time, should not be ruled out. These bacteria show enhanced culturability in the presence of Rpf and most of them do not actively respire. Rpf is required for exit from dormancy by its producer organism, *M. luteus* [28]. By analogy therefore, some of the mycobacterial cells obtained from macrophages may be in a physiological state resem-

bling dormancy. Rpf is a secreted protein, which is believed to act at the cell surface. Growth of extensively washed vegetative (i.e. non-dormant) cells of *M. luteus* in a minimal medium also depends on the addition of exogenous Rpf [28]. Presumably, the secreted (Rv0867c, Rv1884c, Rv2389c and Rv2450c) or membrane anchored (Rv1009) Rpf-like proteins of *M. tuberculosis* are also surface-located, where they would be exposed to the acid hydrolases within phagolysosomes. Their inactivation or removal would account nicely and straightforwardly for the poor culturability and the responsiveness of these cells to exogenously provided Rpf.

After phagocytosis by macrophages, many *M. tuberculosis* cells apparently lose the ability to grow on a solid agar surface. The results reported here show that a liquid medium, supplemented with Rpf, is most appropriate for recovering potentially viable cells of *M. tuberculosis* obtained from macrophages. A similar effect has recently been shown for *M. tuberculosis* H37Ra cells held in prolonged stationary phase in vitro [43]. The beneficial effect of liquid medium on the recovery of *M. luteus* cells that are unable to form colonies on agar-solidified media (e.g. following starvation or thermal stress) has also been reported [9]. The maximum number of viable cells obtained in liquid medium (MPN) in the presence of Rpf was similar in the various different experiments (ca. $10^4$ ml$^{-1}$) and independent of the number of CFU. This value may correspond to the maximum number of potentially viable cells present. Given that the total count (determined microscopically) in the mycobacterial suspensions was ca. $10^6$ cells ml$^{-1}$, it follows that only a few per cent of the bacterial population was recoverable/resuscitable under the conditions employed here. The more pronounced influence of Rpf on culturability (MPN estimated in presence of Rpf) of cell populations with lower CFU (Fig. 2) supports the assumption that the loss of colony-forming ability is connected with accumulation of injured or dormant cells. The small coccoid forms that were not resuscitable represent a significant proportion of the total cell population. Since we cannot be sure that optimal conditions have been employed, permitting maximum effective resuscitation of dormant and/or injured cells, the value of a few per cent resuscitable cells in macrophage lysates represents a lower limit. In addition, although organisms obtained from macrophages appeared to be well-separated unicells, clumping would also lead to under-estimation (though not by 100-fold) of the viable cell number.

The behaviour of *M. tuberculosis* following phagocytosis by macrophages is quite different from that observed using Wayne's model for dormancy, in which *M. tuberculosis* H37Rv cells maintain high viability when they undergo a metabolic shift-down under micro-aerophilic conditions in vitro [12,13]. This transition is associated with cell wall thickening in both pathogenic *M. tuberculosis* and non-pathogenic *M. bovis* BCG strains [44]. The Wayne model may reflect the response of mycobacterial cells to unfavourable conditions as they enter stationary phase. Adaptation to the highly inimical macrophage environment, or persistence of organisms in animal tissues (latency), probably result in the formation of different, uncultivable phenotypes, although whether such cells are dormant, or viable but injured, is not yet clear.

Finally, the observed activity of Rpf with bacterial suspensions obtained from macrophages (substantial increase of MPN counts, decrease of apparent lag phase) suggests that one or more of the cognate *M. tuberculosis* proteins could be involved in mechanisms of latency and reactivation of *M. tuberculosis* in vivo. Rpf or other proteins related to it might also be useful for diagnostic purposes; they may reduce the time to detection of *M. tuberculosis* and decrease the incidence of 'false negatives' in clinical samples.

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